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Investigation of NAA and NAAG dynamics underlying visual stimulation using MEGA-PRESS in a functional MRS experiment

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Abstract

N-acetyl-aspartate (NAA) is responsible for the majority of the most prominent peak in ¹H-MR spectra, and has been used as diagnostic marker for several pathologies. However, ~10% of this peak can be attributed to N-acetyl-aspartyl-glutamate (NAAG), a neuropeptide whose release may be triggered by intense neuronal activation. Separate measurement of NAA and NAAG using MRS is difficult due to large superposition of their spectra. Specifically, in functional MRS (fMRS) experiments, most work has evaluated the sum NAA + NAAG, which does not appear to change during experiments. The aim of this work was to design and perform an fMRS experiment using visual stimulation and a spectral editing sequence, MEGA-PRESS, to further evaluate the individual dynamics of NAA and NAAG during brain activation. The functional paradigm used consisted of three blocks, starting with a rest (*baseline*) block of 320 s, followed by a *stimulus* block (640 s) and a *rest* block (640 s). Twenty healthy subjects participated in this study. On average, subjects followed a pattern of NAA decrease and NAAG increase during stimulation, with a tendency to return to basal levels at the end of the paradigm, with a peak NAA decrease of $-(21 \pm 19)\%$ and a peak NAAG increase of $(64 \pm 62)\%$ (Wilcoxon test, $p < 0.05$). These results may relate to: 1) the only known NAAG synthesis pathway is from NAA and glutamate; 2) a relationship between NAAG and the BOLD response.

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Keywords

Proton magnetic resonance spectroscopy; N-acetyl-aspartate; N-acetyl-aspartyl-glutamate; Visual stimulation; Functional experiments; MEGA-PRESS

1. Introduction

N-acetyl-aspartate (NAA) is one of the most highly concentrated molecules in the central nervous system (CNS) [1]. Due to its prominent signal in MR spectra, NAA has been used as a diagnostic marker for many neuropathologies, including Canavan disease, ischemia, stroke, Alzheimer disease, epilepsy, brain tumors, multiple sclerosis, traumatic brain injury and schizophrenia. Most of these pathologies, with the exception of Canavan disease, have a decreased NAA peak in the MR spectrum. At first this was interpreted as irreversible neural loss. However, there is currently evidence that decreased NAA concentration may also be associated with mitochondrial dysfunction, which in some cases may be reversible. In the particular case of patients with Canavan disease, which is a demyelinating pathology [2], NAA levels in the CNS are increased due to the absence of the enzyme aspartoacylase (ASPA), which is responsible for breaking down NAA [1]. This has suggested that high NAA levels in the CNS may also have harmful effects.

N-acetyl-aspartyl-glutamate (NAAG) is the most highly concentrated dipeptide in the brain [1], and largely localized to neurons. Although NAA is responsible for the largest contribution to the MRS peak located at 2 ppm, NAAG can contribute 10 to 20% of the signal that is often attributed only to NAA [3,4]. Decreases of total NAA associated with neurological disease either involve a joint decrease of NAA and NAAG levels, or may underestimate the decrease of NAA levels, in the case where the NAAG levels increase or remain constant [1]. As mentioned, measurement of the separate contributions of NAA and NAAG using MRS is difficult due to the large superposition of their spectra. To separate these contributions, post-processing methods such as LCModel [5] have been used [3,6,7], however the efficacy of such an approach is variable [8]. Recently Edden et al. used a MEGA-PRESS pulse sequence to separate the contribution of these metabolites in a standard (not dynamic) MRS experiment [9]. On the other hand, in a recent functional MRS (fMRS) experiment with visual stimulation performed by our group, we found a 20% decrease of the NAA signal and a 200% increase of the NAAG signal with stimulus, while their sum remained constant [10]. Few studies have reported similar results [11,12], which have been contested by others [13]. These results were obtained using LCModel, which is likely to be impacted by linewidth changes associated with metabolite BOLD, so in the present work we sought to design and perform an fMRS experiment using the MEGA-PRESS sequence, to further evaluate the individual dynamics of NAA and NAAG underlying brain activation, and contribute to the elucidation of their functions in the nervous system.

2. Subjects, materials and methods

2.1. Subjects

Twenty healthy subjects (mean age 27 ± 6 , range 20–40 years, 8 women) participated in this study. The project was approved by the local ethics committee. Informed consent was obtained from all individual participants included in the study.

2.2. fMRS experiment

The fMRS paradigm consisted of a rest (*baseline*) block (320 s, 20 spectra), followed by a *stimulus* block (640 s, 40 spectra) and a *rest* block (640 s, 40 spectra), with a total duration of 1600 s (around 27 min, totaling 100 spectra) (Fig. 1). The visual stimulus was a black-white radial checkerboard pattern flickering at 8 Hz. This was programmed using the E-Prime software (Psychology Software Tools, USA, www.pstnet.com), and was shown to the subjects on a monitor inside the MR scanner using the Eloquence system (InVivo, USA, www.invivocorp.com).

2.3. MR parameters

MR data were acquired in a 3 T MR system (Achieva, Philips, The Netherlands) with an 8-channel SENSE head coil. Before the fMRS scan, T2-weighted images in three orthogonal planes were acquired, followed by an fMRI protocol with the same visual stimulus used for the fMRS experiment. In the fMRI sequence care was taken to avoid excessive heating of the system, thereby avoiding eventual resonance frequency drifts. Specifically, the maximum number of slices, maximum gradient amplitude and maximum slew rate were reduced resulting in approximately 50% reduction of gradient utilization compared to a standard fMRI sequence. The fMRI acquisition parameters were: EPI sequence with TR/TE = 2000/30 ms, FOV = $240 \times 240 \times 90 \text{ mm}^3$, voxel size = $3 \times 3 \times 3 \text{ mm}^3$, SENSE factor (AP) = 2.5, total scan duration 2 min 28 s. The activation map was superposed on the T2 images, and the MRS voxel was positioned on the occipital lobe over the activated area (Fig. 2). A preparation MRS sequence was run next, for quality control of the standard NAA + NAAG peak in 2 ppm, with parameters: PRESS sequence with TR/TE = 2000/140 ms, 2048 data points, 2000 Hz spectral width, 16 averages and voxel size $3 \times 3 \times 2 \text{ cm}^3$. Finally, the fMRS acquisition took place, using the MEGA-PRESS sequence with the same parameters as the preparation sequence, with exception of the number of spectral averages (which in this case was 8 instead of 16). The MEGA-PRESS editing pulses were set at 4.38 and 4.84 ppm to obtain NAA spectra, and at 4.15 and 4.61 ppm to obtain NAAG spectra [9]. There was a delay of around 2 min between the fMRI and the fMRS sequence.

2.4. MRS data processing

The editing-OFF and -ON spectra were acquired in an interleaved way, shot-by-shot. The intention by doing this was to minimize frequency shifts between corresponding -ON and -OFF spectra. Spectra were always acquired following the same order, first an editing-OFF, then an -ON, and so on. All spectra were apodized (3 Hz) and frequency corrected, using an in-house Matlab routine that used a correlation function to find the maximum of the absolute spectrum over the region of the NAA peak. However, to avoid introducing possibly larger

frequency shift errors between corresponding -OFF and -ON spectra, only editing-OFF (odd) spectra were used to find the frequency shift, and the same frequency shift applied to the odd spectrum was applied to the corresponding editing-ON (even) spectrum [14]. Odd spectra were then subtracted from corresponding even spectra, resulting in edited spectra with an NAA (or NAAG) peak at ~2.5 ppm (Fig. 3). Next, spectra were window-averaged every 20 spectra (320 s), with a step of 10 spectra, that is, averages were done from the first spectrum to the 20th spectrum, then, from the 11th to the 30th spectrum, and so on, until the 81st to the 100th spectrum, for every subject. This last procedure resulted in nine spectra per subject (at nine time points). Finally, the height of the NAA (or NAAG) peak in the real spectra was evaluated. Although the usual choice for evaluating metabolite concentrations is the peak integral, the height was used instead because earlier tests showed that the latter was more stable. All steps were done in Matlab.

2.5. Statistical Analysis

Given the sample size it could not be assumed that the population was normally distributed, therefore a Wilcoxon test was performed to evaluate significant alterations in metabolite concentrations in the *stimulus* and *rest* blocks compared to the *baseline* block. The level of significance used was 5%.

3. Results

Frequency shifts found during frequency correction of the spectra were within typical drift levels, being at most 12.3 Hz for NAA spectra and 18.4 Hz for NAAG spectra, still below the bandwidth of the MEGA-PRESS inversion pulse (which was 40 Hz). Due to poor quality (edited peaks were not visible), NAA data from one subject (subject 10), and NAAG data from two subjects (subjects 4 and 6) were discarded. The signal-to-noise ratio (SNR) was evaluated for all nine spectra corresponding to one of the nine time points evaluated, for the remaining subjects (19 subjects for NAA and 18 for NAAG). The lowest SNR values were 28 for NAA and 24 for NAAG. For these subjects, metabolite levels in all eight spectra corresponding to *stimulus* and *rest* blocks, were compared to the 1st spectrum (*baseline*), and were found to be significantly different for both NAA and NAAG (Wilcoxon test, $p < 0.05$). Most (18 out of 19) subjects followed a pattern of NAA variation consisting of a decrease of NAA with the stimulus, followed by increase after cessation of the stimulus, showing a trend to return back to baseline levels. Also, most (14 out of 18) subjects followed an inverse NAAG variation pattern along the acquisition. For these, NAAG increased with stimulus and decreased after cessation of the stimulus, showing a trend to return back to baseline levels after stimulus cessation. Fig. 4 shows minimum NAA (top) and maximum NAAG (bottom) percentage changes at the *stimulus* block for all subjects, and percentage change values at the end of the acquisition. Minimum NAA values during stimulation ranged from -4 to -73% (Fig. 4, top), while maximum NAAG values ranged from -17 to +173% (Fig. 4, bottom). The average patterns of variation for NAA and NAAG (for all subjects) are shown in Fig. 5, where the error bars represent the standard deviation over subjects. The average minimum change for NAA was $-(21 \pm 19)\%$ (Fig. 5, top), while the average maximum change for NAAG was $(64 \pm 62)\%$ (Fig. 5, bottom).

To make sure that the changes found at 2.5 ppm were not due to subtraction errors, fluctuations at 2 ppm, which are indeed, subtraction artifacts, were also evaluated (also calculating the peak height at that point), and the percentage variations of these fluctuations with respect to the first time point of the peak at 2.5 ppm were computed. The variation pattern found for the subtraction artifact at 2 ppm seemed to be inversely correlated to the variations at 2.5 ppm for most subjects. Therefore, these values were used to estimate a correction for the variations found at 2.5 ppm. These estimated corrections decreased the variations found for NAA and NAAG. Nevertheless, a Wilcoxon test showed that there still remained significant changes ($p < 0.05$) for these metabolites at 2.5 ppm. Fig. 6 shows the corrected average variations for NAA and NAAG (for all subjects), respectively. In these figures, error bars are as for Fig. 5, and significant changes are marked with an asterisk (*). The average minimum change for NAA that was significant was $-(18 \pm 30)\%$ (Fig. 6, top), while the average maximum change for NAAG that was significant was $(56 \pm 90)\%$ (Fig. 6, bottom).

4. Discussion

The aim of the present work was to evaluate the individual dynamics of NAA and NAAG underlying brain activation, and to contribute to the elucidation of their functions in the nervous system.

Although the function of NAA in the nervous system is not yet well known, it has been hypothesized that it acts as a molecular pump, removing excess water created by neuronal energy metabolism out to the extracellular environment [11,15]. However, no protein acting to transport NAA and water out of neurons has yet been characterized. Furthermore, little is known about the propagation mechanisms that specifically regulate NAA release from neurons. As far as it is known, NAA does not have the necessary characteristics for the water pump function [1]. Another function attributed to NAA is that it is an acetate source for myelin lipids synthesis in oligodendrocytes [2]. In addition, it is well known that NAA is associated to the neuronal energetic metabolism, since many experiments in which this metabolism was, intentionally or due to illness, compromised, showed decrease in the levels of NAA [1]. On the other hand, no study has yet been performed that demonstrates a direct link between the synthesis of NAA and of ATP [1]. However, the syntheses of these molecules are at least indirectly related, since the NAA-acetyl is obtained from acetyl-coenzyme-A, whose synthesis, in turn, results from glucose metabolism [1,4].

NAAG is released at synapses and acts as a modulator of the release of neurotransmitters such as glutamate, GABA and dopamine, among others [2]. Release of NAAG depends on the level of neural activity, where intense neuronal activation may result in release of large quantities of NAAG [2]. A role for NAAG has also been suggested in the vascular hyperemic response responsible for the BOLD signal [16]. This last aspect was observed in an experiment in which anesthetized rats were injected with 2-PMPA, an inhibitor of NAAG peptidase, the enzyme responsible for the hydrolysis of NAAG. In these rats, there was an initial increase in BOLD signal relative to baseline levels, followed by a decrease that persisted for several minutes [16]. This effect was explained in terms of NAAG efflux in neurons, its hydrolysis in astrocytes, and the hyperemic oxygenation responses in the brain

[16]. Prior to this, Baslow et al. had raised the hypothesis that NAA and NAAG could have a signaling function between the axon and glia [16,17]. They proposed that these compounds and their intermediates acetate, aspartate and glutamate, are recycled between neuron and glia as a mechanism of intercellular signaling [17]. This model is based on the fact that although NAA and NAAG are synthesized primarily in neurons, their corresponding catabolic enzymes are located in oligodendrocytes and astrocytes, respectively. Thus, the products resulting from the breakdown of NAA and NAAG would be transferred back to neurons and there used in the resynthesis of these compounds [1]. This last part of the hypothesis goes against existing evidence that the acetate and aspartate derived from NAA catalysis in the brain are not used in NAA resynthesis [18]. However, this does not invalidate the hypothesis that NAA and NAAG have a signaling function between neurons and glia, for which there is some additional evidence [19].

There are few works in the literature that have presented results regarding NAA and NAAG variations using fMRS. Indeed, as previously mentioned, we know of only two papers that have shown significant results for NAA variation [11,12], which follow similar patterns to the one found here. These experiments, performed at 1.5 T [12] and 3 T [11], used long stimulation periods (~25 min in [12] and 10 min in [11]), similar to the one used here. Sarchielli et al. [12] found a decrease in NAA levels during visual stimulation for three groups of subjects (patients with migraine with aura, patients with migraine without aura, and control subjects), whereas for patients with migraine with aura this variation was more pronounced (14.61%). This led the authors to suggest that mitochondrial function is less efficient in those patients, since NAA is an indicator of functional integrity of neuronal mitochondrial metabolism [2]. Baslow et al. found a similar decrease to NAA at the end of the stimulus period (13.1%), and explained this decrease through the hypothesis that NAA works as a molecular water pump [11]. So apparently, the results found here agree, at least in pattern, with Refs. [11] and [12]. However, it is important to note that in fact, the NAA signal referred to in Refs. [11] and [12] is most probably the sum NAA + NAAG, since there is no mention in either paper to a separation method for these metabolites.

In addition, most works in the fMRS literature have found no changes in NAA levels. In particular, Mangia and Tack contested [13] Baslow et al. results [11], citing as a counterexample of their own work in a 7 T field [20]. In this work [20], despite the fact that they used LCModel to quantify their spectra, and therefore they could, in principle, have separated NAA from NAAG, it seems that their mention to NAA refers to the NAA + NAAG signal. The work [20] was performed with a paradigm and stimulus similar to Baslow et al. [11], and they found around 2% NAA changes that they attributed to T2 changes due to activation [21]. Although there are some differences between both works, Mangia's group stated that the divergence between the results should not exist [13]. Baslow et al. countered [22], saying that the way Mangia et al. performed the spectral averages in Ref. [20] did not allow finding an NAA variation greater than 2%, and that Mangia et al. [20] took for granted that the NAA signal remained stable, rather than considering this a hypothesis to be verified.

In the present work, we found a decrease in NAA levels in 18 out of 19 subjects, and an increase in NAAG levels in 14 out of 18 subjects. On average, subjects presented a peak

decrease of NAA of $-(21 \pm 19)\%$ and a peak increase of NAAG of $(64 \pm 62)\%$. Even after correcting for subtraction errors at 2 ppm, significant changes of $-(18 \pm 30)\%$ for NAA and $(56 \pm 90)\%$ for NAAG remained. These results fall into the literature discussion outlined above, and therefore may have either of (or a combination of) two explanations. The first explanation would be that the shape of the NAA peak changes due to the T2 changes during regional activation (linewidth narrows and amplitude increases [21]), causing a variation of the height of the curve, which does not imply a concentration change [20,21]. However, there are many points that make this explanation difficult to adapt to our results: 1) T2 changes effects should amount to $\sim 2\%$ variation [20], while the variations found here range from around -4 to -73% ; 2) NAA peak heights should increase [21], and here (measuring peak heights) we found a decrease; 3) T2 changes effects do not explain NAAG changes either, since these are much larger (around -17 to $+173\%$) than the expected effect of 2% , and also, works that have reported T2 changes effects have only done so for the most prominent MRS peaks (water, NAA and creatine [21]). It is worth noting that, although free aspartate has a relatively similar spin system to NAA and NAAG, and it is closely involved with cellular energy metabolism, it does not substantially coedit with the editing scheme used (see e.g. Fig. 3a of [9]).

The second explanation would be that there is indeed a decrease in NAA levels, since NAA contributes for NAAG synthesis [4,23]. However, this is difficult to evaluate, since not all NAA will necessarily transform into NAAG. Upon stimulation, both NAA and NAAG are released to the extracellular fluid at similar rates of about $6\%/h$ [23]. NAA is targeted to oligodendrocytes, which are the only cell type that can catabolize NAA; NAAG consists of a non-excitotoxic form of the neurotransmitter glutamate that is targeted to astrocytes [23]. On the other hand, the formation of an adduct of NAA and glutamate, from which NAAG is synthesized from an NAAG synthase, is the only metabolic pathway available for NAA in neurons [23]. Either way, we used the assumption that all NAA would transform to NAAG and computed the (maximum NAAG change at stimulation)/(minimum NAA change at stimulation) ratio to perform an estimate of the NAA/NAAG concentration ratio. We found a mean ratio¹ of (4.09 ± 3.76) , with values ranging from 0.44 to 12.28 (Table 1). This result falls close to the values of NAA/NAAG concentration ratio estimated in the whole brain of ~ 5 to 10 [3,4], but it stays far apart from the value of 49 reported by Battistuta for the visual cortex of rats [24].² The production of NAAG during neuronal activation agrees with the fact that this metabolite is a neuropeptide, which is liberated in synapses and acts as modulator for the liberation of some neurotransmitters [2]. It also agrees with models for energy metabolism that point to this metabolite as related to the vascular hyperemic response that causes the BOLD signal [4,15].

Finally, when comparing the results of our work with those of the other works cited here, our results apparently agree with those of Baslow et al. [11] and Sarchielli et al. [12], both of which reported decreases in the NAA peak associated with the stimulus, which corresponds qualitatively to our results for this metabolite. It should be remembered, however, that both

¹Considering maximum NAAG and minimum NAA changes for every subject.

²To the best of our knowledge there are no experiments that evaluated this ratio in the human visual cortex.

works effectively measured the joint variation of NAA + NAAG, whereas our methodology allowed us to separate the contributions of these metabolites.

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All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Fig. 1.

Functional activation paradigm. During the *stimulus* block, subjects were visually stimulated with a radial black-and-white checkerboard pattern flickering at 8 Hz.

Fig. 2. MRS voxel positioning. The blue, bolder square is centered on the NAA signal, while the white, lighter square is centered on the water signal.

Fig. 3.

Example of NAA and NAAG edited real spectra for one subject. The nine spectra for each metabolite correspond to the averaged spectra calculated using a sliding window of 20 spectra with a 10-spectra step. The edited peaks at ~2.5 ppm are highlighted.

Fig. 4. Minimum NAA (top) and maximum NAAG (bottom) percentage change during stimulus and return value at the end of paradigm for all subjects (subject 10 was excluded from NAA data and subjects 4 and 6 were excluded from NAAG data due to poor quality data). S1: subject 1, S2: subject 2 etc.

Fig. 5.

Average NAA and NAAG percentage changes (excluding subject 10 for NAA data and subjects 4 and 6 for NAAG data). The values were computed from spectra averaged using a sliding window. The darker blue shaded area represents values obtained from spectra within the stimulation period, while the lighter blue shaded areas represent values obtained from mixing spectra within and without the stimulation period. The non-shaded areas represent values obtained from spectra either in the *baseline* or in the *rest* period. Error bars represent standard deviation over subjects. All changes (8 time points) were significantly different from *baseline* (1st time point) (Wicoxon test, $p < 0.05$).

Fig. 6.

Average NAA and NAAG percentage changes (excluding subject 10 for NAA data and subjects 4 and 6 for NAAG data) after correcting for fluctuations at 2 ppm. Shaded areas and error bars are as in Fig. 5. Asterisks indicate a significant change compared to *baseline* (1st time point).

Table 1

Absolute ratio between maximum percentage changes for NAAG and minimum percentage changes for NAA during stimulation.

Subject	Max NAAG/Min NAA
1	9.39
2	4.40
3	3.90
4	–
5	1.04
6	–
7	8.32
8	0.44
9	5.85
10	–
11	1.61
12	12.28
13	2.93
14	0.95
15	1.93
16	9.52
17	1.35
18	3.10
19	0.87
20	3.74
Mean $\pm$ Std	4.09 $\pm$ 3.76
Maximum	12.28
Minimum	0.44